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SUMMARY. The H5N1 type A influenza viruses classified as Qinghai-like virus (clade 2.2) are a unique lineage of type A influenza viruses with the capacity to produce significant disease and mortality in gallinaceous and anseriform birds, including domestic and wild ducks. The objective of this study was to determine the susceptibility and pathogenesis of chickens and domestic ducks to A/Whooper Swan/Mongolia/224/05 (H5N1) high pathogenicity avian influenza (HPAI) virus when administered through respiratory or alimentary routes of exposure. The chickens and ducks were more susceptible to the H5N1 HPAI virus, as evidenced by low infectious and lethal viral doses, when exposed by intranasal as compared to alimentary routes of inoculation (intragastric or oral-fed infected chicken meat). In the alimentary exposure pathogenesis study, pathologic changes included hemorrhage, necrosis, and inflammation in association with virus detection. These changes were generally observed in most of the visceral organs of chickens, between 2 and 4 days postinoculation (DPI), and are similar to lesions and virus localization seen in birds in natural cases or in experimental studies using the intranasal route. Alimentary exposure to the virus caused systemic infection in the ducks, characterized by moderate lymphocytic encephalitis, necrotized hepatitis, and pancreatitis with a corresponding demonstration of virus within the lesions. In both chickens and ducks with alimentary exposure, lesions, virus, or both were first demonstrated in the upper alimentary tract on 1 DPI, suggesting that the alimentary tract was the initial site affected upon consumption of infected meat or on gavage of virus in liquid medium. However, as demonstrated in the infectivity study in chickens, alimentary infection required higher exposure doses to produce infection as compared to intranasal exposure in chickens. These data suggest that upper respiratory exposure to H5N1 HPAI virus in birds is more likely to result in virus infection and transmission than will consumption of infected meat, unless the latter contains high doses of virus, as found in cannibalized infected carcasses.

RESUMEN. Las diferentes vías de inoculación tienen un impacto en la infectividad y en la patogénesis de la infección por el virus de la influenza aviar de alta patogenicidad subtipo H5N1 en pollos y en patos domésticos.

Los virus de la influenza tipo A, subtipo H5N1 clasificados como del tipo Qinghai (clado 2,2) son un linaje único de los virus de la influenza con la capacidad para producir enfermedad y mortalidad significativas en aves gallináceas y anseriformes, incluyendo los patos domésticos y silvestres. El objetivo de este estudio fue determinar la susceptibilidad y la patogénesis de los pollos y los patos domésticos para el virus de influenza aviar de alta patogenicidad A/cisne cantor/Mongolia/224/05 (H5N1), cuando fue administrado a través de rutas de exposición respiratorias o digestivas. Los pollos y los patos fueron más susceptibles a los virus de la influenza aviar de alta patogenicidad H5N1, según lo evidenciado por dosis virales infecciosas y letales bajas, cuando se expusieron las aves por vía intranasal, en comparación con las rutas digestivas de la inoculación (intragástrica u oral mediante la alimentación con carne de pollos infectados). En el estudio de la patogénesis por exposición digestiva, las alteraciones patológicas incluyeron hemorragias, necrosis e inflamación asociados con la detección del virus. Estos cambios se observaron generalmente en la mayoría de los órganos viscerales de los pollos entre dos y cuatro días posteriores a la inoculación y son similares a las lesiones y a la distribución del virus que se observa en aves con la infección natural o en los estudios experimentales utilizando la vía intranasal. La exposición al virus por vía digestiva causó la infección sistémica en los patos, que se caracterizó por encefalitis linfocitaria moderada, hepatitis necrotizante y pancreatitis con la correspondiente distribución del virus dentro de las lesiones. En los pollos y en los patos con la exposición digestiva, se demostraron la presencia de lesiones, del virus, o ambos inicialmente en el primer día después de la infección, lo que sugiere que el aparato digestivo fue el sitio afectado inicialmente por el consumo de carne infectada o por la administración del virus en un medio líquido. Sin embargo, como se demuestra en el estudio de infectividad en pollos, la infección digestiva requiere de dosis de exposición mayores para producir la infección, en comparación con la exposición intranasal en pollos. Estos datos sugieren que la exposición al virus de la influenza aviar de alta patogenicidad subtipo H5N1 por el tracto respiratorio superior de las aves, es más probable que induzca la infección y transmisión viral en comparación con el consumo de carne infectada, a menos que ésta contenga altas dosis de virus, tal como se encuentra en las canales infectadas que sufrieron canibalismo.

Key words: avian influenza, chickens, ducks, H5N1, infectivity, pathogenesis

Abbreviations: AI = avian influenza; BHI = brain-heart infusion; CID_{50} = mean chicken infective dose; CLD_{50} = mean chicken lethal dose; DID_{50} = mean duck infectious dose; DLD_{50} = mean duck lethal dose; DPI = days postinoculation; EID_{50} = mean embryo infective dose; H&E = hematoxylin and eosin; HPAI = high pathogenicity avian influenza; IG = intragastric; IHC = immunohistochemical; IN = intranasal; LP = low pathogenicity avian influenza; SPF = specific-pathogen-free; REU = relative equivalent units; RRT-PCR = real-time reverse transcriptase–polymerase chain reaction; WL = white leghorn

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Avian influenza (AI) is caused by infection with type A orthomyxoviruses (11,33). Low pathogenicity avian influenza (LPAI) viruses have been isolated from numerous wild and domestic

avian species (33), and wild waterfowl are regarded as the primordial reservoir hosts of these viruses (25,27,35). By contrast, high pathogenicity avian influenza (HPAI) viruses arise from mutation of LPAI viruses as they circulate in poultry, and HPAI viruses are not maintained in wild waterfowl as a primary reservoir (23). HPAI viruses produce severe systemic disease with near 100% mortality in chickens, turkeys, and other gallinaceous birds, but usually have not caused infection, clinical disease, or death in domestic waterfowl or wild birds, especially in aquatic birds of the order Anseriformes (ducks, geese, swans) (1,10,13,19). Interestingly, since the isolation of H5N1 HPAI virus in 1996 from a domestic goose in Guangdong Province, China, descendants of this virus have evolved to express varying capacities to infect and cause disease in domestic ducks and wild aquatic birds under natural and experimental settings (12,15,19).

Most experimental studies on pathogeneses in chickens and ducks have used the intranasal route of exposure and have typically demonstrated initial replication, along with cellular necrosis, in the respiratory epithelium of the nasal cavity followed by virus spread into the submucosa, with infection of capillary endothelium, and then systemic dissemination of the virus to multiple visceral organs (28). Theoretically, avian species have been infected through respiratory tract exposure in the field, but some mammals, including dogs (26) and cats (14), have been infected through the gastrointestinal tract following consumption of infected poultry, other birds, or their products (9). Ferrets and pigs have been infected following high-dose experimental oral or alimentary exposure to H5N1 HPAI virus-infected chicken meat (16,17). In addition, a human case with H5N1 HPAI viruses was linked to the ingestion of uncooked duck blood (8).

There is little information on the pathogenesis of infections in chickens and domestic ducks following alimentary tract exposure and on the dose required to induce such infection. One study indicated that scavenging gulls can be infected by consumption of infected raw poultry products (5) and, in some situations, poultry may be exposed by scavenging dead carcasses or by consuming infected products. In this study, we present the pathologic changes, the immunohistochemical (IHC) distribution of H5N1 viral antigen, and an assessment of the basic pathogenesis of the viral infection and disease in chickens and ducks following oral ingestion or direct intragastric inoculation of H5N1 HPAI-infected chicken meat. In addition, to assess susceptibility, we compare the relative dose of virus required to produce infection via the respiratory versus alimentary tracts in chickens and ducks.

MATERIALS AND METHODS

Virus propagation. The challenge HPAI virus, A/Whopper Swan/Mongolia/244/05 (H5N1; Mongolia/05), was used as a second chorioallantoic sac passage from 10-day-old embryonating chicken eggs. Allantoic fluid from inoculated eggs was collected and diluted 1:300 in brain-heart infusion medium (BHI). Similarly, a sham inoculum was made with sterile allantoic fluid diluted 1:300 in BHI.

Animals. Four-week-old, specific-pathogen-free (SPF) white leghorn (WL) chickens (*Gallus domesticus*) and 2-to-3-wk-old conventional domestic ducks (*Anas platyrhynchos*) were used in this study. Serum samples were collected from five randomly selected birds of each species, prior to inoculation, to ensure that the birds were serologically negative for AI virus as determined by the agar gel precipitin test. The birds were housed separately in self-contained isolation units (Mark IV, Controlled Isolation Systems, San Diego, CA) that were ventilated under negative pressure with inlet and exhaust HEPA-filtered air and maintained under continuous lighting. Feed and water were provided *ad libitum*. General

care was provided as required by the Institutional Animal Care and Use Committee, as outlined in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching. All experiments were performed in a U. S. Department of Agriculture certified biosafety level-3 enhanced facility at the Southeast Poultry Research Laboratory.

Determination of infectivity by different routes of inoculation. The first study, done to determine infectivity of HPAI virus for chickens by different exposure routes and in different media, used groups of 3–5 birds inoculated with different virus doses and by different routes of inoculation as follows: 1) into the nasal cavity (intranasal; IN) via choanal slit with virus in liquid media (IN-liquid), 2) intragastric (IG) with virus in liquid media (IG-liquid), 3) oral consumption of virus-infected meat (meat-fed), 4) intragastric with virus-infected meat (IG-meat), and 5) oral consumption of virus in water (Oral-liquid). The chickens in the IN- and IG-liquid groups received 0.1 ml of inoculum containing 10^1, 10^3, and 10^5 mean embryo infective doses (EID_{50}) or 0.5 ml of inoculum containing 10^3, 10^5, 10^7, and 10^8 EID_{50}, respectively. IG-meat chickens received 10^6, 10^7, or 10^8 EID_{50} of virus in 0.01g, 0.1g, or 5g of meat, respectively. Individually housed chickens consumed between 0.2 and 1.8 g of meat, equivalent to exposures of $10^{5.0}$ to $10^{7.6}$ EID_{50} (meat-fed). For Oral-liquid group, chickens consumed between 8 and 14 ml of the HPAI virus-contaminated water ($10^{5.5}$ EID_{50}/ml). All birds were then monitored daily for clinical signs of disease or death until 14 days after exposure.

Groups of three ducks were challenged, with three different doses of the virus, in three different inoculation groups: 1) IN inoculation of liquid through choanal slit ($10^{1.9}$, $10^{3.7}$, and $10^{5.7}$ EID_{50}/0.1 ml), 2) IG inoculation in liquid by gavage with syringe and cannula ($10^{5.8}$, $10^{6.8}$, and $10^{7.8}$ EID_{50} in 0.5 ml), and 3) IG inoculation with minced infected chicken meat by gavage with syringe and cannula ($10^{5.9}$, $10^{6.9}$, and $10^{7.9}$ EID_{50} in 0.01 to 1g of infected meat). All birds were then monitored daily for clinical signs of disease or death until 14 days after exposure.

Infection in individual birds was determined by detection of AI virus matrix gene by real-time reverse transcriptase–polymerase chain reaction (RRT-PCR) in oropharyngeal or cloacal swabs from 1–14 days postinoculation (DPI) or by seroconversion in survivors based on agar gel immunodiffusion tests at 14 DPI. The RRT-PCR results were determined as relative equivalent units based on a standard curve of challenge virus infectious titers. The mean chicken and duck infectious and lethal doses were calculated by the method of Reed and Muench (24).

Pathogenesis study. The goal of the second study was to determine, through alimentary exposure routes, how disease pathogenesis differed from previously published studies using IN exposure. The chickens and ducks were fed meat via manual placement in the mouth or were given virus-contaminated liquid via crop gavage to produce alimentary tract exposure. The latter exposure was done because ducks play in water, resulting in excessive losses of water to the environment and making quantification of virus exposure impossible.

For each species, birds were separated into a control group and a virus-inoculated group. The control group contained two birds from each species that were IG-inoculated with 1 ml of the sham inoculum by gavage. The two control chickens and ducks were euthanatized on the same day when the last virus-inoculated bird died or was euthanatized.

For virus-inoculated groups, the chickens and ducks were separated into the following four experimental groups: 1) IG-liquid inoculated chickens – 10 chickens IG-inoculated with 1 ml of inoculum containing 10^8 EID_{50} of the virus through a plastic cannula, 2) meat-fed chickens – 10 chickens fed 0.5 g of meat from chickens infected with Mongolia/05 virus (meat titer, $10^{7.6}$ EID_{50}); for each chicken, the meat was placed in the mouth and the beak was gently held closed until the birds swallowed, 3) IG-liquid inoculated ducks – 10 ducks IG-inoculated with 1 ml of inoculum containing $10^{7.2}$ EID_{50} of the virus through a plastic cannula, and 4) meat-fed ducks – 10 ducks fed 1 g of chicken meat infected with Mongolia/05 virus ($10^{7.9}$ EID_{50}). All birds were then monitored daily for clinical signs of disease. Oropharyngeal and cloacal swabs were collected in 1.5 ml of BHI media with antibiotics from all inoculated birds at 1, 2, 3, 4, and 5 DPI, except

that the last sampling time for meat-fed ducks was at 7 rather than 5 DPI. A minimum of two randomly selected birds of each group were euthanatized and necropsied at 1, 2, 3, 4, and 5 or 7 DPI or as birds became moribund or died. Gross lesions were recorded for each bird and tissues were collected for histopathologic examination.

All control and virus-inoculated birds were humanely euthanatized by intravenous administration of sodium pentobarbital (100 mg/kg body weight).

Histopathology and immunohistochemical staining. Collected tissues were fixed by submersion in 10% neutral buffered formalin, routinely processed, and embedded in paraffin. Sections were made at 5 μm and were stained with hematoxylin and eosin (H&E). A duplicate 5-μm section was IHC stained with a mouse-derived monoclonal antibody (P13C11) specific for type A influenza virus nucleoprotein antigen as the primary antibody (21). Procedures for IHC followed those previously described. Fast red was used as a substrate chromogen and slides were counterstained with hematoxylin. Demonstration of viral antigen was based on chromogen deposition in the nucleus, which was often accompanied by chromogen deposition within the cytoplasm.

Virus detection. RNA was extracted from oropharyngeal or cloacal swab samples as follows: 250 μl of BHI from the swab sample was added to 750 μl of Trizol LS (Invitrogen Inc., Carlsbad, CA). The sample was mixed by vortexing and incubated at room temperature for 10 min and then 200 μl of chloroform was added. The samples were mixed by vortexing, incubated at room temperature for 10 min, and then centrifuged for 15 min at approximately $12{,}000 \times g$. The aqueous phase was collected, and RNA isolation was completed by extracting the RNA from the aqueous phase with the AI/ND viral RNA isolation kit (MagMAX®; Ambion, Inc., Austin, TX) in accordance with the kit instructions and using a magnetic particle processing system (King-Fisher®; Thermo Scientific, Waltham, MA).

The AI virus challenge strain was used to produce the RNA for the quantitative standard. Allantoic fluid virus stocks were diluted in BHI broth (Becton-Dickinson, Sparks, MD) and titrated in 10-day-old embryonating chicken eggs at the time of dilution as per standard methods (29). Whole virus RNA was extracted from 10-fold dilutions of titrated virus as described for swab material. Quantitative RRT-PCR for the influenza matrix gene was performed. Virus titers in samples were calculated, based on the standard curves, by the Smart Cycler II® (Cepheid, Inc. Sunnyvale, CA) software or by extrapolation of the standard curve equation. Samples with cycle threshold values over 38 were considered suspect and were confirmed as positive or negative by conventional RT-PCR for the NS1 gene, followed by gel electrophoresis. Because the NS1 test is not quantitative, titer values for the NS1-positive samples were calculated based on the matrix gene test. RRT-PCR results were reported as relative equivalent units (REU) based on the standard curve.

RESULTS

Infectivity test in chickens and ducks. Chicken and ducks were inoculated by different routes of exposure, but the dose required to produce infection differed with the route of exposure and the medium containing the virus. With IN inoculation of HPAI virus in liquid, mean chicken infective dose (CID_{50}) was $10^{3.3}$ for individually housed chickens, which is similar to the previous CID_{50} of $10^{2.8}$ EID_{50} for chickens that were group housed (6). When inoculated by IG-liquid route, chickens required higher inoculation doses to produce infections; i.e., a CID_{50} of $10^{6.2}$ EID_{50} and $10^{6.3}$ EID_{50}, for individual versus group-housed chickens, respectively. When the HPAI virus was contained in infected meat, oral consumption of meat (meat-fed) or IG exposure to infected meat by gavage (IG-meat) produced CID_{50}s of 10^{7} EID_{50} and $\geq 10^{7.4}$ EID_{50}, respectively. When chickens were allowed to drink HPAI virus-contaminated water, the CID_{50} was $10^{6.7}$ EID_{50}. These results indicate that, to produce infection, upper respiratory exposure requires an approximately 10^{3-4} EID_{50} lower dose of virus than does alimentary tract exposure. The doses by drinking or gavage were similar in producing infections; however, if the virus was in liquid given directly into the crop, it required a $\log_{10}$ lower dose to produce infection than if the virus were in infected meat. The CID_{50} and the mean chicken lethal dose (CLD_{50}) were identical for respective chicken study groups, except for the IG-meat which, by gavage, had $CLD_{50} > 10^{7.9}$ EID_{50}.

In ducks, the four different exposure routes produced infection. Interestingly, the virus caused significant mortality (2 or 3 dead of 3 infected birds) via IN, IG-liquid, and IG-meat routes, which were dose-dependent, from 3 to 7 days after infection. The mean duck infectious dose (DID_{50}) for IN-liquid, IG-liquid, and IG-meat was $<10^{1.9}$, $<10^{5.8}$, and $<10^{5.9}$ EID_{50}, respectively. The mean duck lethal dose (DLD_{50}) for IN-liquid, IG-liquid, and IG-meat was $<10^{1.9}$, $<10^{5.8}$, and $10^{6.4}$ EID_{50}, respectively. Although endpoints were not obtained for DID_{50} or DLD_{50}, the ducks were more likely to become infected at a lower challenge dose and to not exhibit the mortality that occurred in the chickens.

Pathogenesis study. *Control groups.* No clinical signs or mortality were observed in the sham-inoculated control chickens or ducks. Grossly and histologically, control birds lacked lesions and all samples were negative for AI viral antigen on IHC staining.

Clinical signs and gross lesions: IG-liquid inoculated and meat-fed chickens. Individual IG–liquid-inoculated chickens presented with mild listlessness, anorexia, ruffled feathers, and mild diarrhea, with mortality as early as 2 DPI. From 3 DPI, they showed severe diarrhea, severe listlessness, and a reluctance to move, even with digital pressure. Three chicken deaths were observed on each of days 2 and 4 postinoculation.

In the IG–liquid-inoculated group, two chickens euthanatized on 1 DPI lacked lesions except for a mild dilation of the small intestine. On 2 DPI, mild focal hemorrhages and necrosis in the comb were seen in a chicken found dead. Mild-to-moderate mucosal hemorrhage at the esophageal–proventricular junction, mild-to-moderate mucosal hemorrhage of the proventricular mucosa, empty and dilated small intestine, moderate pulmonary edema with severe congestion, and mild-to-moderate renomegaly were present in both chicken groups on 2 DPI. By 3 DPI, the two chickens had a mild-to-moderate decrease in body fat, dehydration, and mild congestion with edema in lungs. One chicken had a slight enlargement of the spleen with mild mucosal hemorrhage of the cecal tonsil. In the three dead birds that were found on 4 DPI, hemorrhages were observed in the alimentary tract, such as at the esophageal–proventricular junction, and in the mucosa of the proventriculus, ventriculus, and cecal tonsil.

The meat-fed chickens had clinical signs, mortality patterns, and gross lesions similar to the IG–liquid-inoculated chickens.

Histology and immunohistochemisty: IG–liquid-inoculated and meat-fed chickens. Histologic lesions and the corresponding viral antigen were distributed among multiple tissues. The distribution and average severity of histologic lesions, and the average distribution and frequency of viral antigen, are summarized in Tables 1 and 2.

On 1 DPI, mild heterophilic infiltration in the laminar propria of the proventriculus and small intestine, especially the duodenum (Fig. 1) and jejunum, were noted. From the chickens that were sampled or died between 2–4 DPI, almost every part of the gastrointestinal tracts had degeneration and necrosis of villar and crypt mucosal epithelial cells and mild-to-moderate degeneration of ganglion cells in the myenteric plexus. In particular, on 2 DPI, the esophageal–proventricular junction from dead chickens showed

Table 1. Average distribution of histologic lesions in chickens inoculated via IG-liquid route containing Mongolia/05 virus or orally fed meat (meat-fed) from chickens infected with Mongolia/05 virus. Chickens were sampled on 1, 2, 3, and 4 DPI.

Inoculation route	DPI	Nasal cavity	Tongue	Esophagus	Crop	Esophageal–proventricular junction	Proventriculus	Duodenum and jejunum	Pancreas	Cecal tonsil	Intestinal myenteric plexi	Lung	Heart	Brain	Spleen	Cloacal bursa and thymus
IG-liquid	1	−[A]	NS[B]	NS	−	−	+	±	−	±	−	−	−	−	−	−
	2	+	NS	NS	±	+	++	+	+	+	+	++	+++	++	+++	++
	3	−	NS	NS	±	+	+	+	±+	+	−	+	++	+	+	+
	4	++	NS	NS	±	±	++	+	+++	+	+	+++	+++	+++	+++	+
Meat-fed	1	NS	−	+	+	−	−	±	−	−	−	−	−	−	−	−
	2	NS	+	+	+++	+	++	++	++	++	−	+++	+++	+++	+++	++
	3	NS	+	+	++	+	++	++	++	++	±+	+++	+++	+++	+++	+
	4	NS	+	+	+	+	++	+	++	++	±+	+++	+++	+++	+++	++

[A]Lesion scores: (−) = no lesion; (±) = minimal; (+) = mild; (++) = moderate; (+++) = severe.
[B]NS = no sample.

Table 2. Average distribution of AI viral antigen in the tissues from chickens inoculated via IG-liquid route containing Mongolia/05 virus or orally fed meat (meat-fed) from chickens infected with Mongolia/05 virus. Chickens were sampled on 1, 2, 3, and 4 DPI.

Inoculation route	DPI	Nasal cavity	Tongue	Esophagus	Crop	Esophageal–proventricular junction	Proventriculus	Duodenum and jejunum	Pancreas	Cecal tonsil	Intestinal myenteric plexi	Blood vessels	Lung	Heart	Brain	Spleen	Bursa and thymus
IG-liquid	1	−[A]	NS[B]	NS	−	−	−	−	−	−	−	−	−	−	−	−	−
	2	++	NS	NS	+	+	++	++	+++	++	+	+++	++	+++	++	+++	++
	3	++	NS	NS	+	+	++	++	++	+	+	+++	++	+++	++	+++	+
	4	++	NS	NS	++	++	++	++	+++	++	+	−	+++	+++	+++	+++	+
Meat-fed	1	NS	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	2	NS	+	+	+++	+	++	++	++	++	±	+++	+++	+++	+++	+++	++
	3	NS	+	+	++	+	+	++	+++	++	±	+++	+++	+++	+++	++	++
	4	NS	+	+	+	+	++	++	+	++	±	+++	++	+++	+++	+++	++

[A]AI viral antigen staining: (−) = no antigen staining; (±) = rare; (+) = infrequent; (++) = common; (+++) = widespread.
[B]NS = no sample.
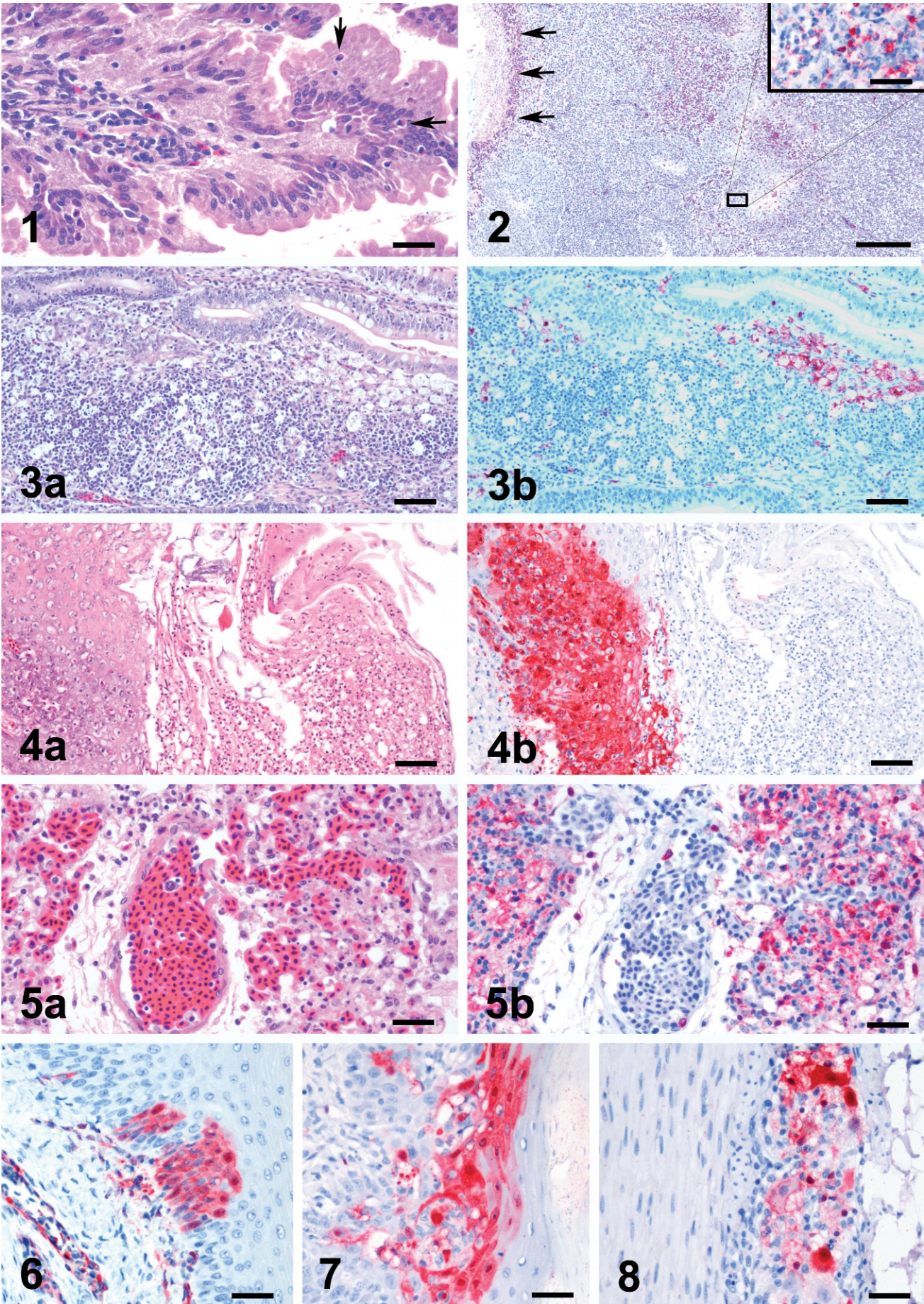
severe epithelial necrosis of the mucosal gland with heterophilic infiltration and hemorrhage. Inflammation and necrosis were more severe in both the proventriculus and cecal tonsils than in other portions of the alimentary tract. The proventriculus had moderate mucosal epithelial necrosis and desquamation, moderate mucosal hemorrhage, mild-to-moderate heterophilic infiltration in the tunica propria, and vacuolar degeneration and necrosis of glandular epithelium; these were prominent on 2 DPI and continued, though slightly less severely, on 3 and 4 DPI. In the cecal tonsils, there was mild-to-moderate necrosis and heterophilic infiltration with exocytosis. Gut-associated lymphoid tissues at the esophageal–proventricular junction, the submucosal lymphoid follicles (''Peyer's patches'') of the small intestine (Fig. 3a), and the cecal tonsils had lymphoid depletion with accompanying necrosis of lymphocytes, many of which contained apoptotic bodies.

The brain had multiple foci of malacia with gliosis and mild-to-moderate lymphocytic perivascular cuffs. The pancreas had multifocal-to-confluent acinar epithelial necrosis with mild heterophilic inflammation. Other lesions present included mild heterophilic inflammation in the nasal cavity, multifocal myocardial necrosis with heterophilic-to-lymphocytic infiltration, severe heterophilic-to-histiocytic interstitial pneumonia, and severe lymphocytic depletion in the spleen due to necrosis, apoptosis, or both, of the periellipsoidal and periarteriolar sheaths accompanied by sinusoidal congestion. Additionally, some areas of spleens had mild-to-moderate, heterophilic-to-histiocytic inflammation that was accompanied by severe lymphoid depletion and moderate necrosis.

In the meat-fed group on 1 DPI, one chicken had extensively focal degeneration, necrosis, or both, of stratified squamous epithelium with moderate heterophilic inflammation around necrotic areas (Fig. 4a) and between the basal cells and tunica propria in the esophagus and crop. Mild heterophilic infiltration in the villar lamina propria, with slight exocytosis, was seen in the duodenum and jejunum. Between 2 and 4 DPI, most tissues and organs sampled from dead or euthanatized chickens had moderate-to-severe cellular degeneration and necrosis with generalized passive congestion, hemorrhage, or both, similar to lesions seen in chickens of the IG-liquid group. Specific lesions seen included the following: 1) moderate-to-severe heterophilic-to-histiocytic interstitial pneumonia, 2) mild-to-severe necrotic rhinitis and multifocal myocardial necrosis, 3) lymphocytic degeneration and necrosis of lymphoid organs in the gastrointestinal tracts from the esophageal–proventricular junction to cecal tonsils, 4) lymphocytic depletion, necrosis, and apoptosis in the thymus and cloacal bursa, 5) moderate-to-severe lymphocytic encephalitis with randomly small foci of gliosis and malacia, and 6) mild, focal epithelial cell necrosis with minimal heterophilic infiltration (Fig. 6a) in the stratum spinosum and basale of the tongue, esophagus, and crop.

In the IG-liquid group, chickens euthanatized on 1 DPI lacked viral antigen in all tissues (Table 2). However, between 2–4 DPI, AI viral antigen was most consistently demonstrated in the majority of endothelial cells of various-sized blood vessels throughout the body. Commonly, viral antigen was detected in the mucosal epithelium, and phagocytic leukocytes were seen in the lamina propria (Fig. 3b) of the digestive tract including proventriculus, duodenum, jejunum, and cecal tonsil; they were less commonly seen in the crop and esophageal–proventricular junction mucosal epithelium. Viral antigen was common-to-widespread in the pancreatic glandular epithelium, in epithelium of the nasal cavity or infraorbital sinuses, in heterophils and histiocytes in the lungs, cardiac myocytes, neurons and glial cells of the brain, and in phagocytes and ellipsoid-associated cells of the spleen. Infrequently, AI viral antigen was seen in the stratified squamous and basal cell epithelium of the tongue (Fig. 6) and crop (Fig. 4b), in autonomic nerves of the myenteric plexi of the lower digestive tracts (especially jejuna and ceca), in macrophages of the thymus and bursa, and in the renal tubular epithelium. The distribution of viral antigen in meat-fed chickens was similar to that of the IG–liquid-inoculated chickens, except that AI antigen was more common-to-widespread in the crop of meat-fed chickens on 2 and 3 DPI.

Clinical signs and gross lesions: IG–liquid-inoculated and meat-fed ducks. No clinical signs or mortality were observed in the IG-liquid group; however, one meat-fed duck was found dead on 2 DPI. The two IG-liquid ducks euthanatized on 1 and 2 DPI lacked gross lesions except for a few small white spots in the spleen and slight renomegaly from one bird on 2 DPI. The meat-fed duck found dead on 2 DPI had mild hemorrhages and congestion of the heart, moderate edema of lungs, and slightly swollen kidneys. On 3 and 4 DPI, the IG-liquid and meat-fed ducks had numerous necrotic foci in the pancreas and whitish, segmented necrosis of the myocardium with hyperpericardium, and the small intestines were moderately dilated with mucus and watery contents. On 5 DPI, two IG-liquid

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Fig. 1.   Duodenum: IG-liquid chicken, 1 DPI. Mild heterophilic infiltration (arrow) in villi of the duodenal mucosa. H&E. Bar = 25 μm.

Fig. 2.   Esophageal–proventricular junction: IG-liquid duck, 1 DPI. Extensively focal necrosis (arrows) of mucosal epitheliums with slight lymphocytic depletion and mild hemorrhage. H&E. Bar = 100 μm. Insert: AI viral antigen detected in necrotized histiocytes. Biotin-streptavidin complex with hematoxylin counterstain. Bar = 25 μm.

Fig. 3.   Jejunum: IG-liquid chicken, 2 DPI. (a). Moderate lymphocytic depletion with lymphocyte cell death in the gut-associated lymphoid tissues with individual enterocytic necrosis. H&E. Bar = 50 μm. (b). AI viral antigen detected in the necrotized lymphocytes, histiocytes, and individual enterocytes. Biotin-streptavidin complex with hematoxylin counterstain. Bar = 50 μm.

Fig. 4.   Crop: meat-fed chicken, 2 DPI. (a). Severe necrosis and degeneration of mucosal epithelium with heterophilic inflammation and desquamation of superficial necrotic epithelial layers. H&E. Bar = 25 μm. (b). AI viral antigen detected in the degenerative and necrotic mucosal epithelium and heterophils. Biotin-streptavidin complex with hematoxylin counterstain. Bar = 25 μm.

Fig. 5.   Lung: meat-fed duck, 2 DPI. (a). Severe histiocytic interstitial pneumonia with moderate congestion and edema. H&E. Bar = 25 μm. (b). AI viral antigen detected in the histiocytes in air capillaries and endothelial cells of blood capillaries and small blood vessels. Biotin-streptavidin complex with hematoxylin counterstain. Bar = 25 μm.

Fig. 6.   Tongue: meat-fed chicken, 2 DPI. AI viral antigen detected in the stratified squamous epithelial cells, basal cells, and vascular endothelium. Biotin-streptavidin complex with hematoxylin counterstain. Bar = 25 μm.

Fig. 7.   Tongue: meat-fed duck, 4 DPI. AI viral antigen detected in the degenerative and necrotic squamous epithelial cells. Biotin-streptavidin complex with hematoxylin counterstain. Bar = 25 μm.

Fig. 8.   Peripheral nerves: meat-fed duck, 4 DPI. AI viral antigen detected in the degenerative nervous fibers in the autonomic ganglia of the digestive tract. Biotin-streptavidin complex with hematoxylin counterstain. Bar = 25 μm.

Table 3. Average distribution of histologic lesions from ducks inoculated via IG-liquid route containing Mongolia/05 virus or orally fed meat (meat-fed) from chickens infected with Mongolia/05 virus. Ducks were sampled on 1, 2, 3, 4, and 5 or 7 DPI.

Inoculation route	DPI	Nasal cavity	Tongue	Esophagus	Crop	Esophageal–proventricular junction	Proventriculus	Duodenum and jejunum	Pancreas	Cecal tonsil	Intestinal myenteric plexi	Lungs	Heart	Brain	Spleen	Cloacal bursa and thymus
IG-liquid	1	−[A]	NS[B]	NS	−	+	−	−	−	−	−	−	−	−	−	−
	2	+	NS	NS	±	+	±	−	+	±	−	+	+	++	++	++
	3	+	NS	NS	−	±	−	−	+	±	+	++	+++	++	−	+
	4	+	NS	NS	−	±	±	+	++	−	+++	+	+++	+	−	+
	5	++	NS	NS	−	−	−	−	−	−	−	−	−	++	−	+
Meat-fed	1	NS	−	−	−	−	−	±	+	−	−	−	−	−	−	−
	2 (E)[C]	NS	+	+	±	+	−	±	+	−	±	+	+	+	+	+
	2 (D)[D]	NS	+	±	−	−	−	±	+	−	+	+++	+	+++	++	++
	3	NS	++	−	±	±	−	±	++	−	++	±	+++	+++	±±	+
	4	NS	−	−	+	±	−	+	±	±	++	−	++	++	−	+
	7	NS	−	−	−	−	−	−	−	−	++	−	−	++	−	±±

[A] Lesion scores: (−) = no lesion; (±) = minimal; (+) = mild; (++) = moderate; (+++) = severe.
[B] NS = no sample.
[C] E = euthanatized duck.
[D] D = dead duck.

Table 4. Average distribution of AI viral antigen in the tissues from ducks inoculated via IG-liquid route containing Mongolia/05 virus or orally fed meat (meat-fed) from chickens infected with Mongolia/05 virus. Ducks were sampled on 1, 2, 3, 4, and 5 or 7 DPI.

Inoculation route	DPI	Nasal cavity	Tongue	Esophagus	Crop	Esophageal–proventricular junction	Proventriculus	Duodenum and jejunum	Pancreas	Cecal tonsil	Intestinal myenteric plexi	Blood vessels	Lung	Brain	Heart	Spleen	Cloacal bursa and thymus
IG-liquid	1	−[A]	NS[B]	NS	±	+	±	−	−	−	−	−	−	−	−	−	−
	2	++	NS	NS	±	±	+	−	++	−	−	−	+++	+++	++	++	+++
	3	+	NS	NS	−	−	−	−	+	−	+	−	++	++	++	−	+
	4	+	NS	NS	−	−	−	−	++	−	±	−	+	+	−	−	±±
	5	++	NS	NS	−	−	−	−	++	−	−	−	−	−	++	−	+
Meat-fed	1	NS	−	−	−	−	−	−	−	−	±	−	−	−	−	−	−
	2 (E)[C]	NS	++	−	±	±	±	−	+	+	+	−	+	++	+++	+	+
	2 (D)[D]	NS	++	±	++	+	+	+	+	−	+	±	+++	+++	++	++	++
	3	NS	+	−	±	−	−	−	+	−	±	−	+	+++	++	−	+
	4	NS	+++	−	+	±	−	−	++	−	++	−	+	+	−	−	+
	7	NS	−	−	−	−	−	−	−	−	−	−	−	+	−	−	±±

[A] Viral antigen scores: (−) = no antigen; (±) = rare antigen; (+) = infrequent antigen; (++) = common antigen; (+++) = widespread antigen.
[B] NS = no sample.
[C] E = euthanatized duck.
[D] D = dead duck.

Table 5. Virus detection in oropharyngeal and cloacal swabs from chickens and ducks on 1, 2, 3, 4, and 5 or 7 DPI with Mongolia/05 (H5N1) HPAI virus given intragastrically in liquid media (IG-liquid) or orally fed Mongolia/05 infected chicken meat (meat-fed).

Species	Infection route	Oropharyngeal swabs (DPI)						Cloacal swabs (DPI)					
		1	2	3	4	5	7	1	2	3	4	5	7
Chickens	IG-liquid	0/2[A]	3/3 (7.7)[B]	2/2 (6.85)	3/3 (8.4)	NA[C]	NA	0/2	3/3 (7.2)	2/2 (5.9)	3/3 (7.2)	NA	NA
	Meat-fed	0/10	4/8 (7.9)	3/4 (7.1)	2/2 (7.1)	NA	NA	0/10	5/8 (7.7)	3/4 (6.8)	2/2 (7.5)	NA	NA
Ducks	IG-Liquid	0/2	1/2 (6.9)	2/2 (6.95)	2/2 (6.4)	2/2 (6.5)	NA	0/2	1/2 (7.1)	2/2 (5.65)	1/2 (5.9)	0/2	NA
	Meat-fed	2/10 (5.8)	4/8 (6.8)	4/6 (6.5)	2/4 (7.1)	NA	0/2	3/10 (6.7)	5/8 (5.2)	1/6 (5.4)	1/4 (5.9)	NA	0/2

[A]No. positive/no. sampled.
[B]Mean quantity of virus for positive samples as determined by quantitative RRT-PCR as reported in REU scaled to $\log_{10}$ EID_{50}/ml.
[C]NA = not applicable; no birds available for sampling.

ducks showed mild-to-moderate atrophy of the cloacal bursa and thymus. No gross lesions were found in meat-fed ducks euthanatized on 7 DPI.

Histology and immunohistochemisty: IG–liquid-inoculated and meat-fed ducks. The IG–liquid-inoculated and meat-fed ducks showed more restricted distribution and less severe histologic lesions and viral antigen than was seen in the corresponding group of chickens. Histologic lesions and the corresponding viral antigen were distributed among multiple tissues. The distribution and average severity of histologic lesions, and the average distribution and frequency of viral antigen, are summarized in Tables 3 and 4.

Only the esophageal–proventricular junction of the IG-liquid ducks had specific lesions on 1 DPI, characterized by extensively focal epithelial necrosis (Fig. 2) and desquamation with heterophilic infiltration and exocytosis, mild degeneration and necrosis of mucous glandular epithelial cells with intraluminal cellular debris, and minimal lymphocytic necrosis. In meat-fed ducks sampled on 1 DPI, the esophagus had minimally focal degeneration of basal cells with minimal lymphocytic infiltration in the lamina propria. On 2–5 DPI, the IG-liquid and meat-fed ducks had similar lesions. Mild mucosal epithelial degeneration and necrosis with minimal heterophilic inflammation were seen in the alimentary tract from the tongue to ceca, with minimal-to-moderate neuronal degeneration and necrosis of ganglia in myenteric plexi accompanied by rare heterophilic-to-mononuclear inflammation. The most significant lesions in the other visceral organs were mild-to-moderate pancreatic acinar epithelial cell necrosis; randomly scattered gliosis with mild-to-severe lymphocytic encephalitis; moderate-to-severe myocardial necrosis with lymphocytic-to-histiocytic inflammation; mild lymphocytic depletion in the thymus and bursa; and minimal histiocytic interstitial pneumonia, with mild mononuclear cellular infiltration around perivascular areas of the lung air capillaries and interlobular spaces, and minimal vacuolar degeneration of bronchiolar epithelial cells. The nasal cavity had minimal mucosal epithelial, glandular epithelial degeneration, and necrosis with minimal heterophilic inflammation. The brain and autonomic ganglia of the myenteric plexi had moderate degeneration and necrosis of neurons, with mild mononuclear cellular infiltration up to 7 DPI. By contrast, the meat-fed duck that died on 2 DPI had more-severe changes in the brain, lungs, spleen, cloacal bursa, thymus, and peripheral nerves as compared to the euthanatized bird in either group. Specifically, the lungs had severe heterophilic-to-histiocytic interstitial pneumonia and moderate edema in the air capillaries and interlobular areas, with mild-to-moderate congestion of most capillaries (Fig. 5a); the primary lymphoid organs had moderate lymphocyte depletion, and severe necrosis in neurons was seen in the brain and autonomic nerves.

In both the IG-liquid and meat-fed ducks, viral antigen demonstration was less frequent and was more restrictive as to organ- and cell-type than was seen in either corresponding groups of chickens. In the IG-liquid ducks, viral antigen was detected in mucosal epithelial cells of the crop and proventriculus and in phagocytes of gut-associated lymphoid tissue from the esophageal–proventricular junction at 1 DPI (Fig. 2). From 2–5 DPI, both IG-liquid and meat-fed ducks that were euthanatized had infrequent-to-common viral antigen in ganglia and nerve fibers of the myenteric plexi (Fig. 8), mucosal epithelium of the nasal cavity, myocardial fibers, neurons and glial cells in the brain, pancreatic acinar epithelial cells, and histiocytes in the cloacal bursa and thymus. On 7 DPI, only the brain had a positive reaction, specifically in neurons, glial cells, Purkinje cells, and rarely, in ependymal cells. A meat-fed duck found dead had more widespread AI viral antigen demonstration than did the euthanatized ducks of either the IG-liquid or meat-fed groups and included stratified epithelium, basal cells, and mucosal glandular epithelial cells in the tongue (Fig. 7), esophagus and crop; glandular epithelial cells in the proventriculus; cardiac myocytes; hepatocytes and Kupffer cells in the liver; pancreatic acinar epithelium; ganglia and nerve fibers of myenteric plexi; heterophils and histiocytes in the lungs (Fig. 5b); and neurons and glial cells in the brain. Most interestingly, there was prominent AI viral staining in the endothelium of blood vessels in the lungs, heart, and rarely, in brain, duodenum, jejunum, and cecal tonsil (Fig. 5b).

Virus shedding. Virus replication and shedding was for <7 DPI and followed the standard pattern associated with infection with an influenza A virus (Table 5). In chickens, the virus titers were very high by 2 DPI for both oropharyngeal and cloacal swabs and remained high until all birds had died on 4 DPI. For the ducks, virus replication peaked on 2 and 3 DPI, for cloacal and oropharyngeal swabs in IG-liquid ducks, respectively, while in the meat-fed group the peak was 1 and 4 DPI, respectively.

DISCUSSION

In this study, 3-wk-old SPF WL chickens and 2-wk-old conventional domestic ducks were used for studies on viral infectivity and pathogenesis in relation to route of exposure. The 50% bird infective dose is a quantitative estimation of infectivity which allows the comparison of different species and viruses, as well as inoculation routes (as reported in prior studies), for an assessment of relative infectivity and adaptation (30). In the present study, we found that the chickens were more susceptible to infection to Mongolia/05 virus in aqueous phase following respiratory versus alimentary exposure, as evidenced by lower CID_{50} from the IN challenge ($CID_{50} = 10^{3.3}$) as compared to the IG-liquid ($CID_{50} = 10^{6.2–6.3}$) or Oral-liquid ($CID_{50} = 10^{6.7}$) challenge. Even exposure to infected meat (meat-fed or IG-meat; $CID_{50} = \geq10^{7.0}$) required an approximately 10^4 higher dose exposure than did IN exposure and a 10^1 higher exposure dose than did virus in liquid for alimentary tract exposure. This is consistent with influenza viruses, which are primarily respiratory in tropism, but

it also emphasizes that alimentary exposure to Mongolia/05 virus in high doses can initiate infection. The IN-exposure dose for Mongolia/05 falls within the range of other HPAI viruses (CID_{50}: median = $10^{3.2}$, range = $10^{1.2}$–$10^{4.7}$) that have caused natural outbreaks in chickens and turkeys (30). For chickens, the mean infectious and lethal doses were higher than that of wood ducks (*Aix sponsa*), $10^{0.95}$ and $10^{1.71}$, respectively (6), and mean infectious doses for house sparrows (*Passer domesticus*), $<10^{2.4}$ (3), indicating a species difference for susceptibility when using the same Mongolia/05 H5N1 HPAI strain with an IN route of inoculation. In a previous study in ferrets and mice, alimentary exposure was less successful at producing H5N1 HPAI virus infection than was IN exposure when using the same dose (15). In the current study in ducks, a lower intranasal virus dose than in chickens was required to produce infection, but the virus produced less-frequent lethal outcomes than seen in chickens.

In our infectivity study, IN inoculation or alimentary exposure in meat or liquid with high doses of the Mongolia/05 HPAI virus in chickens resulted in almost 100% morbidity and mortality within 4 days of inoculation. This result verifies the high pathogenicity of the Mongolia/05 virus for the chickens, irrespective of respiratory versus alimentary exposure routes. Prior studies with intravenous inoculation have produced 100% mortality in chickens.

In the pathogenicity study, the maximal virulence of the Mongolia/05 HPAI virus was seen in chickens rather than in ducks, with earlier onset and more-rapid progression of clinical disease to a uniform lethal outcome. In ducks, the virus was more infectious, as evidenced by a lower mean bird infectious dose of $<10^{1.9}$ in ducks as compared to $10^{2.8}$ in chickens; however, such infections in ducks rarely progressed to clinical disease and death at low challenge doses. Moreover, in the chickens, macroscopic and microscopic lesions for birds exposed by either IG inoculation or oral consumption were dominated by acute congestion, hemorrhage, or both, and edema and necrosis of multiple organs. These lesions were consistent with those of previous IN inoculation studies indicating maximal pathogenicity of HPAI virus isolates for gallinaceous hosts of origin: The infection begins with a local replication in the respiratory tract, invasion and replication in the vascular endothelium, and then systemic spread of the virus via the vascular system with lesion development in multiple organs (18,28,31,37). Because HPAI viruses, rather than the low pathogenicity AI viruses, can replicate in endothelial cells and spread systemically in gallinaceous birds, as occurred in chickens in our pathogenesis study, such viruses can cause tissue damage and death due to ischemia from vascular thrombosis and multiple organ failure (19,20,27,28). Moreover, we demonstrated that alimentary tract exposure in chickens and ducks initiates virus replication in upper alimentary sites, principally the oral cavity, esophagus, crop, and esophageal-proventricular junction, with production of prominent virus-associated lesions, indicating that the alimentary tract can be a primary site of exposure and infection in birds.

In some experimental studies, ducks have been considered to be resistant to both infection and clinical signs from various HPAI viruses, when exposed via the IN-inoculation route, and any mortality observed has been infrequent (1,2,6,15,20,21,33,34). Recently, systemic spread of a few H5N1 HPAI viruses has been demonstrated in some infected ducks (19,20,32). Furthermore, many recent natural and experimental reports have shown the increased pathogenicity and high mortality potential of some strains of the H5N1 HPAI viruses for domestic ducks, waterfowl, and other wild birds (2,4,5,12,19,22). In the present study, we observed that 2-wk-old domestic ducks showed infection and clinical signs with high mortality when given 10^2 EID_{50} of Mongolia/05 virus by IN route, but the alimentary routes of

challenge required higher doses to produce infection, disease, or death. Also, based on the pathogenesis test, systemic spread of Mongolia/05 H5N1 virus to various internal organs, including brain, heart, lung, pancreas, and peripheral nerves, was produced by alimentary tract exposure in ducks. These findings are in contrast to previous high-dose IN-inoculation studies with the H5N1 viruses isolated before 2002, which primarily replicated in the respiratory and alimentary tracts but without causing mortality, and virus localization and lesions in visceral organs was rare (21). Londt *et al.* (18) suggested that increased pathogenicity in ducks might have resulted from some mutation into virulence for ducks at the time of the 2005 emergence of the Lake Qinghai isolates. However, the 2002 Hong Kong H5N1 viruses from captive waterfowl have demonstrated high lethality for domestic ducks in experimental studies (28).

In contrast to chicken and other gallinaceous species, in which vascular damage produced severe pulmonary edema, congestion, and microthrombosis, as well as common reports of viral antigen demonstration in the vascular endothelium, waterfowl (including domestic ducks) have rarely been described with such histologic changes or viral antigen in blood vessels when infected with HPAI viruses (7,15,18,31). By contrast, in our study, one duck infected by the meat-fed route, and that died at 2 DPI, had widespread vascular damage with antigen detected in the endothelium of capillaries, small arteries, and veins in various visceral organs including the lungs, heart, and rarely, brain, duodenum, jejunum, and cecal tonsil. Such vascular lesions could explain the death of this duck, and the lack of such vascular lesions might have been associated with survival in the other ducks.

Intranasal inoculation of chickens with H5N1 HPAI virus initiates a local infection of the nasal cavity epithelium, with necrosis and invasion of mucosa and capillary endothelial cell replication, followed by a systemic spread of the virus (28). In the present pathogenesis study, IG-liquid exposure initiated infection in the alimentary tract, with lesions on 1 DPI in the esophageal–proventricular junction, proventriculus, duodenum, and cecal tonsil in chickens and ducks, followed by a systemic virus spread and lesions in multiple visceral organs and brain on 2 DPI. The IG-liquid group of ducks had virus demonstrated in the crop, esophageal–proventricular junction, and the proventriculus on 1 DPI. Furthermore, lesions seen in the meat-fed groups suggested that, in chickens and ducks, the initial site affected was the crop and esophageal–proventricular junction, respectively, although IHC demonstration of virus replication in the upper digestive tract was absent on 1 DPI. By contrast, in other studies, feeding pigs with Mongolia/05-infected chicken meat initiated a respiratory infection through oropharyngeal exposure and tonsil infection, but without evidence of alimentary infection and with no systemic virus replication or lesions (17). In ferrets, feeding meat from chickens infected with respiratory trophic A/Muscovy duck/Vietnam/209/05 (H5N1) and Mongolia/05 (H5N1) viruses produced infection that was limited to the respiratory tract and was initiated through the tonsil (17). However, when A/Vietnam/1203/2003 (H5N1) HPAI virus, a strain that caused systemic infection in ferrets, was fed as infected chicken meat, the ferrets became infected and died, with initiation of infection through both the respiratory and alimentary tracts.

Our studies suggest that alimentary exposure of chickens and ducks with Mongolia/05 HPAI virus can initiate an enteric infection and that the virus can spread systemically. However, alimentary exposure required a 10^4 higher dose of virus than did IN exposure to produce infection. The initiation of H5N1 HPAI virus infection in birds is still favored by a respiratory route of exposure, via respiratory droplets and airborne fomites such as dust, but alimentary initiated infection is possible if birds consume high doses of virus, such as occurs when cannibalizing infected carcasses.

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